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Kamata et al., Microbiol., Immunol., 38(6):421-428, 1994,

Varon et al., J. of Neurotrauma 11(5):473-486, 1994,

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Microbiol. Immunol., 38(6), 421-428, 1994

Morphological Effects, Rate of Incorporation, and the Enzymatic Action of Botulinum ADP-Ribosyltransferase, Known as C3 Exoenzyme, on Human Neuroblastoma GOTO Cells

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Received December 17, 1993; in revised form, February 2, 1994. Accepted February 8, 1994

***Abstract*: The susceptibility of various lines of cultured cells to botulinum ADP-ribosyltransferase, known as C3 exoenzyme, was examined. Human neuroblastoma GOTO cells were most sensitive. The C3 exoenzyme caused a change in cell shape that involved extension of neurites. The exoenzyme evoked the outgrowth of neurites from chick ganglion as effectively as nerve growth factor, suggesting that C3 exoenzyme possesses neurotropic activity. Experiments with ^{125}I-labeled enzyme revealed that C3 exoenzyme was rapidly incorporated into cells but the number of incorporated enzyme molecules was small. Once C3 exoenzyme had been incorporated, ADP-ribosylation of the substrate (Rho protein) in GOTO cells occurred immediately and rapidly reached a maximum level. However, some of Rho proteins remained unmodified even after induction of the change in morphology. These findings suggest that ADP-ribosylation by C3 exoenzyme is directly associated with the differentiation of GOTO cells but that other events may also participate in this process.**

Key words: **Botulinum ADP-ribosyltransferase, Neuroblastoma cells, Neurite formation, Incorporation**

Clostridium botulinum type C and D strains produce a highly potent neurotoxin, a cytotoxin called C2, and an ADP-ribosyltransferase known as exoenzyme C3 (1, 19). C3 exoenzyme has been purified as a protein with a molecular mass of 23 kDa (2, 12). C3 exoenzyme catalyzes the ADP-ribosylation of small GTP-binding proteins with molecular masses of about 21 to 24 kDa (11, 18). The substrates of C3 exoenzyme have been identified as the products of *rho* gene, members of the *ras* superfamily of proteins (3, 6, 13, 16). This ADP-ribosylation occurs at a specific asparagine residue in the putative effector domain of Rho proteins and presumably affects the cellular function (20). Treatment of Vero cells with C3 exoenzyme induces a morphological change and the disassembly of actin filaments (21). Therefore, it appears that Rho proteins are involved in regulating the organization of polymerized actin. C3 exoenzyme, when introduced into PC-12 cells, causes changes in the morphology of the cells that are accompanied by formation of neurite-like extension and an increase in the activity of acetylcholine esterase (14). From these results, it appears that C3 exoenzyme, which ADP-ribosylates Rho proteins, might be useful as a promising agent for an examination of the functions of Rho protein. Compared with other bacterial toxins with ADP-ribosylation activity or with other biological agents such as hormones and growth factors, the action of C3 exoenzyme requires a relatively high concentration of the enzyme for detection of its action on intact cells (14, 18). No attempt has been made to deter-

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Abbreviations: ADP, adenosine 5′-phosphate; DMEM, Dulbecco's modified Eagle's medium; ED_{50}, 50% effective dose; EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum; GTP, guanosine 5′-phosphate; IgG, immunoglobulin G; MEM, minimum essential medium; NAD, nicotinamide adenine dinucleotide; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

mine quantitatively how much C3 exoenzyme is taken up by intact cells. In this study we examined the potential of C3 exoenzyme for use as a neurotropic agent and we performed a quantitative assay to determine what extent C3 exoenzyme is incorporated into human neuroblastoma GOTO cells. We also attempted to clarify the correlation between the C3 exoenzyme-induced change in morphology and ADP-ribosylation of Rho proteins in the cells.

Materials and Methods

Botulinum C3 exoenzyme. Clostridium botulinum type C strain 003-9 was used for purification of C3 exoenzyme as described in detail elsewhere (2). The exoenzyme was purified from a culture supernatant by ammonium sulfate precipitation, chromatography on CM-Sephadex (Pharmacia LKB Bioproducts, Tokyo), and gel filtration on Sephadex G-100 (Pharmacia). The exoenzyme was then applied to a Mono S HR 5/5 (Pharmacia) column that had been equilibrated with 0.05 M Tris-HCl buffer, pH 7.0, and it was eluted with a linear gradient of NaCl from 0 to 0.3 M. After dialysis against 0.1 M phosphate buffer, pH 7.5, the purified exoenzyme (100 μg) was radioiodinated with Na ^{125}I (0.9 mCi; Amersham Life Science, Tokyo) by the chloramine T method (8). ^{125}I-labeled C3 exoenzyme was separated from free ^{125}I by gel filtration on a column of Sephadex G-50 that had been equilibrated with Dulbecco's modified Eagle's medium (DMEM; Gibco Laboratories, Grand Island, N.Y., U.S.A.) that contained 10% fetal calf serum (FCS; M.A. Bioproducts, Walkersville, Md., U.S.A.). ^{125}I-labeled C3 exoenzyme had a specific radioactivity of 8.0 mCi (296 MBq)/mg protein. It was sterilized by passage through a membrane filter (0.22 μm) before use. ADP-ribosylation activity of C3 exoenzyme was not affected after iodination.

Treatment of cultured cells with C3 exoenzyme. GOTO cells were grown in DMEM that contained 10% FCS. PC-12 cells were grown in DMEM that contained 5% FCS and 5% horse serum (Gibco). Vero, LLC-MK2, HeLa, and FL cells were maintained in Eagle's minimum essential medium (MEM) that contained 0.03% L-glutamine and 8% newborn calf serum (Gibco). To determine the morphological effects of C3 exoenzyme, cells (2×10^4/cm^2) were plated on culture plates or dishes and incubated at 37 C for 18 to 48 hr. Then the cells were cultured for the indicated times in fresh medium that contained C3 exoenzyme at various concentrations. The effects of C3 exoenzyme on the cells were expressed in the rate of the number of cells that changed morphologically among more than 200 cells examined in each case. The cells with dendrites were counted as morphologically changed cells.

Neurite-outgrowth assay. Sympathetic ganglia were dissected from 8-day chick embryos according to the method of Ebendal (7). A ganglion was placed in a 35-mm collagen-coated culture dish and incubated with MEM that contained 10% FCS and 0.03% L-glutamine in the presence of C3 exoenzyme. β-Nerve growth factor (Sigma Chemicals Co., St. Louis, Mo., U.S.A.) was used as a positive control. After 24 hr at 37 C, neurite outgrowth from the ganglion was examined.

Incorporation of ^{125}I-labeled C3 exoenzyme. GOTO cells were cultured in DMEM that contained 10% FCS in the presence of ^{125}I-labeled C3 exoenzyme. After incubation, the cells were washed five times with Dulbecco's phosphate-buffered saline (PBS) without Mg^{2+} and Ca^{2+} ions [PBS (−)]. The washed cells were then treated with 0.025% trypsin (Difco) in PBS (−) at 37C for 5 min. After washing again with PBS (−), the cells were counted and solubilized at 37 C for 10 min in 0.5 ml of 50 mM Tris-HCl buffer, pH 7.4, that contained 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA; Wako Pure Chemicals, Osaka, Japan), 5 mM KI, 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma), 10 μg/ml pepstatin (Peptide Institute, Osaka, Japan), 10 μg/ml chymostatin (Peptide Institute), 10 μg/ml leupeptin (Peptide Institute), 10 μg / ml aprotinin (Sigma), 1 μg/ml *o*-phenanthroline (Sigma), 10 μM benzamidine (Sigma), 0.02% NaN_3 and 0.05% Nonidet P-40 (Wako). After subsequent reaction with rabbit polyclonal antibodies raised against C3 exoenzyme (5 μg in 50 μl), prepared in our laboratory, 10% *Staphylococcus aureus* Cowan I cells (0.1ml) were added. After incubation at 4 C for 30 min, the Cowan I cells were washed twice by centrifugation with 50 mM Tris-HCl buffer, pH 7.4, that contained 150 mM NaCl, 5 mM EDTA, 5 mM KI, 0.02% NaN_3, 0.05% Nonidet P-40 and 1% bovine serum albumin (Sigma). Finally, the radioactivity of the pellet was determined with a gamma counter (Cobra B50033; Packard Instrument Co., Dowers Grove, Ill., U.S.A.).

To examine the fate of ^{125}I-labeled C3 exoenzyme that had been incorporated in the cells, the solubilized suspension of cells was mixed with trichloroacetic acid (TCA) at a final concentration of 15%

TCA. The precipitate was redissolved in 20 mM Tris-HCl buffer, pH 6.8, that contained 2% sodium dodecyl sulfate (SDS), 1% 2-mercaptoethanol and 20% glycerol, and then it was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on a 12.5% gel. The gel was stained with Coomassie brilliant blue and dried. ^{125}I-labeled C3 exoenzyme was visualized by autoradiography with Kodak X-Omat AR film.

ADP-ribosylation assay. ADP-ribosylation activity was assayed as described previously (15). Cells were homogenized in 0.01 M phosphate buffer, pH 8.5. The homogenate (25 μg of protein) was combined in a total volume of 50 μl of reaction buffer that contained 0.1 M Tris-HCl buffer (pH 8.5), 50 mM nicotinamide, 10 μM [^{32}P]NAD (1.25 μCi; Dupont, NEN Research Products) with C3 exoenzyme (50 ng). The reaction mixture was incubated at 37 C for 45 min and the reaction was terminated by the addition of TCA at a final concentration of 10%. The precipitate was solubilized in the presence of 1% 2-mercaptoethanol and 1% SDS, and then it was subjected to 12.5% SDS-PAGE. After staining of the gel with Coomassie brilliant blue, autoradiography of the dried gel was carried out as described above. The incorporated radioactivity was quantified by excising radioactive bands from the gel and measuring the radioactivity in Scintisol

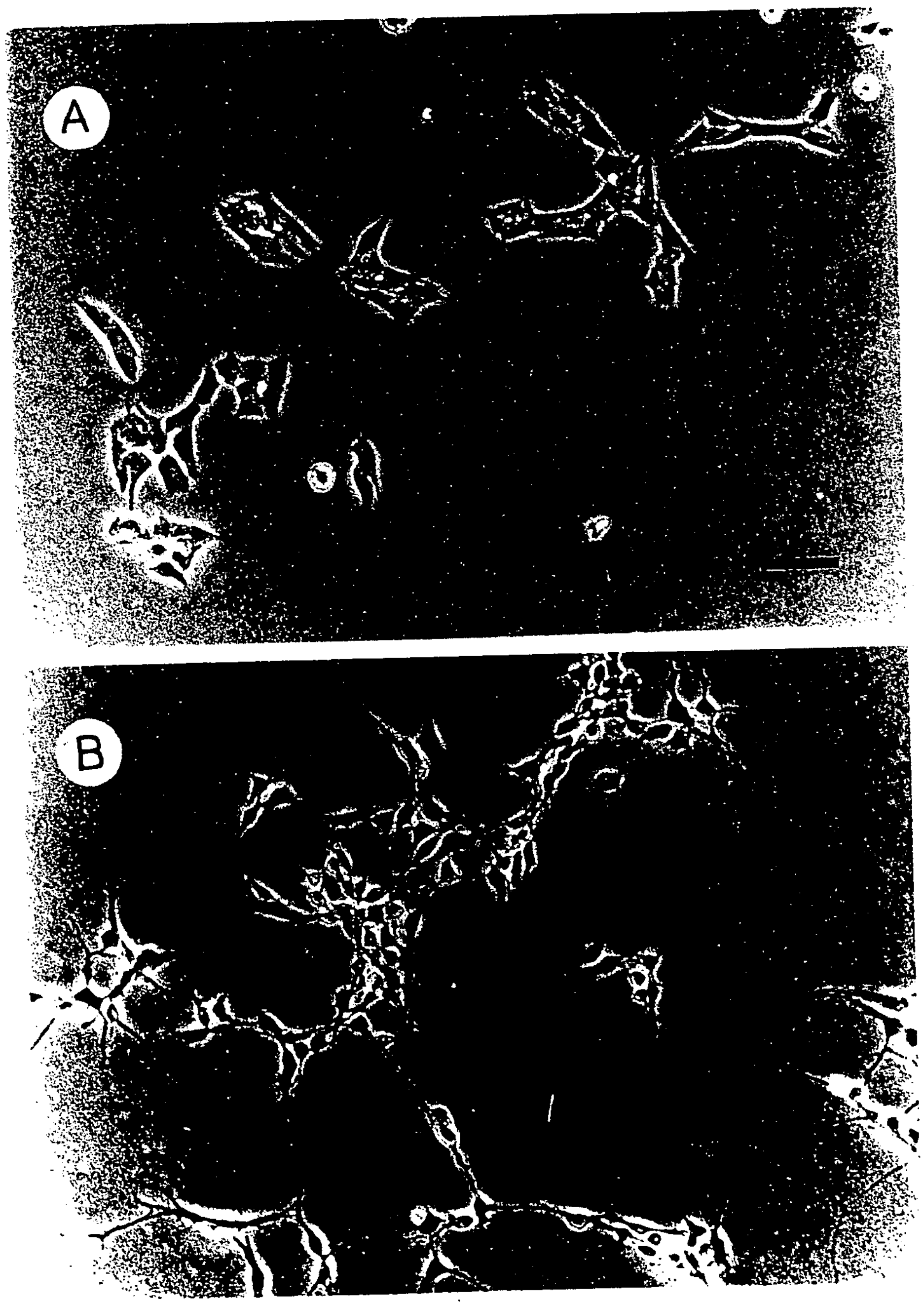

Fig. 1. Morphological changes in GOTO cells cultured in the absence (A) and in the presence (B) of botulinum C3 exoenzyme. GOTO cells were incubated with C3 exoenzyme at 10 μg/ml for 24 hr at 37 C in DMEM that contained 10% FCS. Bar shows 200 μm.

EX-H (Wako).

Other methods. Proteins were quantified by the method of Lowry et al (10). Rabbit polyclonal antibodies (IgG) against C3 exoenzyme were purified by affinity chromatography on a column of Affi-gel 10 (Bio-Rad Laboratories, Tokyo) that had been coupled with C3 exoenzyme. SDS-PAGE was carried out by Laemmli's method (9).

Results

Effects of C3 Exoenzyme on the Morphology of Cultured Cells

When cultured cells were treated with C3 exoenzyme at 10 μg/ml, cells of all lines tested changed their shape, with characteristic rounding up of cell bodies, condensation of the cytosol, and the appearance of dendrites. The cultured cells were distinctly susceptible to C3 exoenzyme at 50% of the effective dose (Table 1). Human neuroblastoma GOTO cells were the most sensitive among the tested lines, and Vero, LLC-MK2 and PC-12 cells were moderately affected by C3 exoenzyme. HeLa and FL cells were less sensitive: when these cells were treated with C3 exoenzyme at 100 μg/ml, only 30% of the cells of each line changed in shape. In GOTO cells, condensation of the cytosol was much marked and cells became visibly dendritic (Fig. 1). As previously reported (14), formation of short neurites was observed in the case of PC-12 cells treated with C3 exoenzyme. To confirm the neurite-inducing activity of C3 exoenzyme, the neurite-outgrowth assay was performed with chick ganglia. As shown in Fig. 2, C3 exoenzyme evoked the formation of neurites at a relatively high concentration of the exoenzyme as compared with the effective concentration of nerve growth factor.

C3 exoenzyme caused morphological changes in GOTO cells in a dose-dependent manner (Fig. 3).

Table 1. Susceptibility of different cell lines to botulinum C3 exoenzyme

Cell line	Origin	ED_{50}(μg/ml)
GOTO	Human neuroblastoma	0.3
PC-12	Rat pheochromocytoma	45
Vero	Monkey kidney	3.6
LLC-MK2	Monkey kidney	7.0
HeLa	Human cervicoma	>100
FL	Human amnion	>100

Cells (2×10^4/cm^2) were incubated in medium that contained C3 exoenzyme at various concentrations. After a 24-hr incubation at 37 C, the shapes of cells were examined and then the 50% effective dose (ED_{50}) for each cell line was obtained from a dose-response curve.

The appearance of dendrites was detectable with C3 exoenzyme at 30 ng/ml after 24-hr incubation, with 100% of cells being affected at 3 μg/ml. When GOTO cells were observed periodically after the addition of C3 exoenzyme at 3 μg/ml to the medium, morphological changes were detectable within 1 hr (Fig. 4). GOTO cells that had been pulse-treated with C3 exoenzyme were cultured for a total of 24 hr in fresh medium without C3 exoenzyme and then their shapes were examined. Pulse-treatment with C3 exoenzyme for 5 hr caused all the cells to become dendritic. Thus, a 5-hr

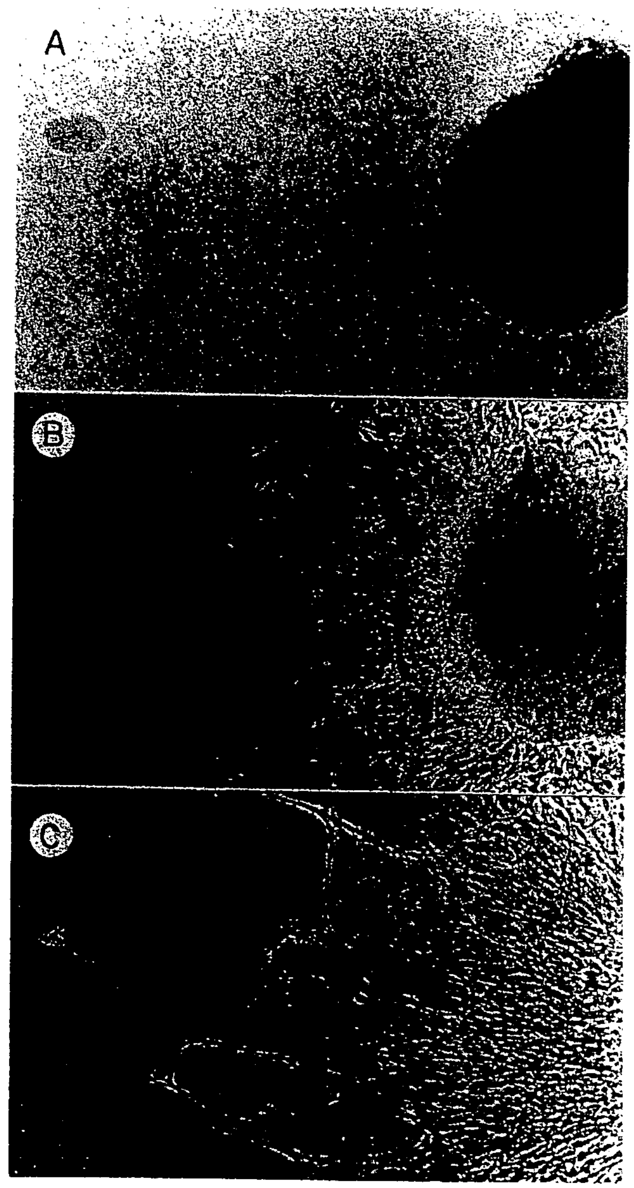

Fig. 2. Effects of botulinum C3 exoenzyme and nerve growth factor on chick embryonic ganglia. A ganglion was cultured in the absence (A) and in the presence of C3 exoenzyme (B, 50 μg / ml) or nerve growth factor (C, 1 μg/ml). Bar shows 200 μm.

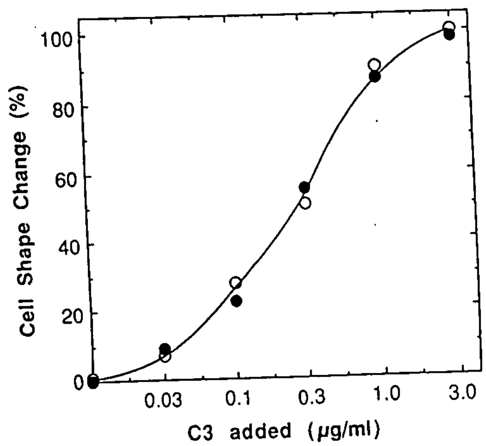

Fig. 3. Dose-dependent effects of botulinum C3 exoenzyme (○) and [125]I-labeled exoenzyme (●) on the rate of the changes in shape of GOTO cells. The cells ($2 \times 10^4/cm^2$) were incubated for 24 hr in the presence of C3 exoenzyme at the indicated concentrations. Cells with alteration in morphology were counted among more than 200 cells in each case.

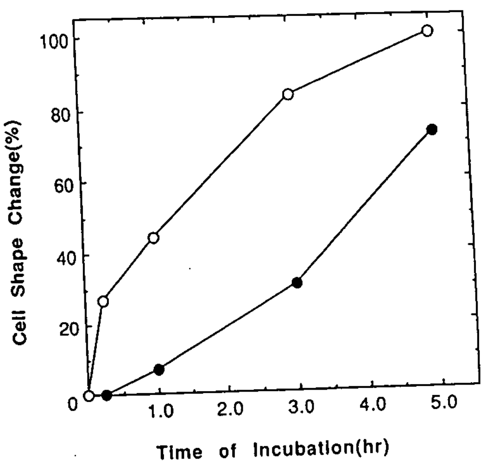

Fig. 4. Time-dependent effects of botulinum C3 exoenzyme on the rate of the change in shape of GOTO cells. The cells were treated with C3 exoenzyme at 3 μg/ml for the indicated times and immediately the number of cells with altered morphology was counted (●). After pulse-treatment with C3 exoenzyme for indicated times, the cells were washed and cultured for a total of 24 hr in DMEM that contained 10% FCS without C3 exoenzyme. Cells with altered morphology were counted (○).

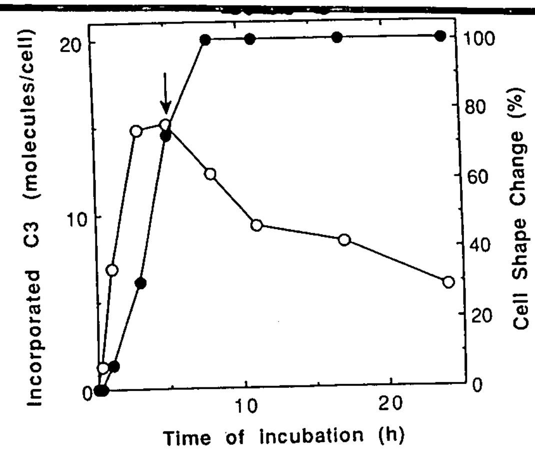

Fig. 5. Time course of the change in morphology of GOTO cells and the incorporation of botulinum C3 exoenzyme. GOTO cells were incubated in the presence of 3 μg/ml [125]I-labeled C3 exoenzyme for the indicated times at 37 C. Then the rate of changes in cell morphology (●) and the number of incorporated molecules of C3 exoenzyme (○) were determined. The cells were washed with fresh DMEM that contained 10% FCS at 5 hr (arrow) and then the incubation was continued for a total of 24 hr.

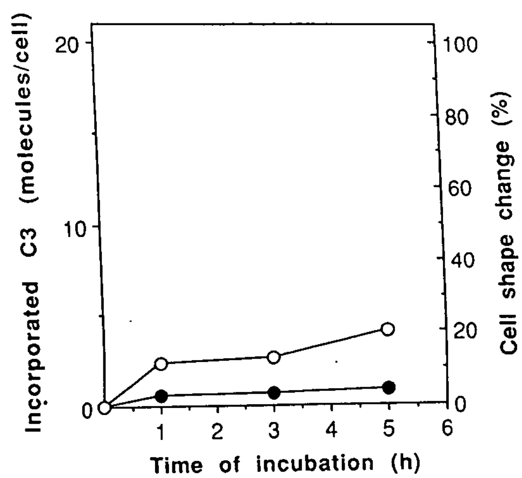

Fig. 6. Morphological effects on and incorporation of botulinum C3 exoenzyme by GOTO cells at 4 C. GOTO cells were incubated in the presence of [125]I-labeled C3 exoenzyme at 3 μg/ml for the indicated times. After incubations, the rate of changes in morphology (○) and the number of incorporated molecules of C3 exoenzyme (●) were determined.

incubation with C3 exoenzyme was sufficient to induce a morphological change in GOTO cells.

Characterization of C3 Exoenzyme Incorporated into GOTO Cells

First we examined the biological activity of C3 exoenzyme after iodination. [125]I-labeled exoenzyme retained all of the original activity of the exoenzyme when assayed with GOTO cells, as shown in Fig. 3, and it entered the cytosol of GOTO cells in a concentration-dependent manner during a 24-hr incubation at 37 C. The number of molecules of C3 exoenzyme incorporated by GOTO cells was determined periodically. C3 exoenzyme appeared to be entrapped rapidly by GOTO cells but the number of molecules of the incorporated C3 exoenzyme by each cell was small (Fig. 5). During a 1-hr incubation, approximately 7 molecules of C3 exoenzyme were counted per individual GOTO cell. About 70% of GOTO cells changed in shape in 5 hr at 37 C in C3

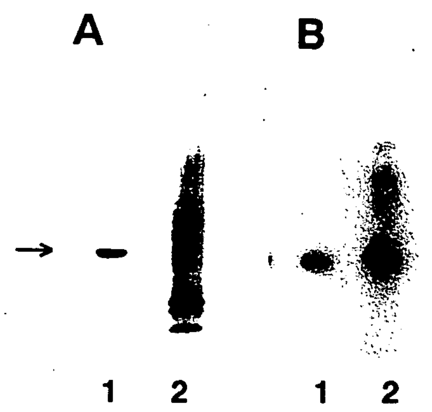

Fig. 7. Analysis of botulinum C3 exoenzyme that had been incorporated into GOTO cells. Staining with Coomassie brilliant blue (A) and autoradiography (B) after electrophoresis of a gel that had been loaded with ^{125}I-labeled C3 exoenzyme (lane 1) and a lysate of GOTO cells that had been treated for 5 hr with ^{125}I-labeled C3 exoenzyme (lane 2). See "Materials and Methods" for details.

exoenzyme-containing medium and at this point 15 molecules of C3 exoenzyme were detected per GOTO cell (Fig. 6). At 4 C, by contrast, only 20% of GOTO cells showed a change in cell shape and approximately 1 molecule of C3 exoenzyme was found per cell (Fig. 6).

^{125}I-labeled C3 exoenzyme migrated during electrophoresis as a position with molecular mass of 23 kDa, as did the unlabeled exoenzyme. No degraded products were found among the molecules of ^{125}I-labeled C3 exoenzyme that had been incorporated into GOTO cells (Fig. 7).

ADP-Ribosylation in GOTO Cells with C3 Exoenzyme

C3 exoenzyme ADP-ribosylated proteins with a molecular mass of approximately 21 kDa in homogenates of GOTO cells. These proteins probably corresponded to Rho proteins (Fig. 8). ADP-ribosylation in GOTO cells by C3 exoenzyme occurred in a dose-dependent manner. The amount of unmodified substrate protein in GOTO cells was quantified. GOTO cells contained 5 pmol per mg protein of potential substrate for ADP-ribosylation (Fig. 9). The ADP-ribosylation seemed to occur rapidly after treatment with C3 exoenzyme and reached a maximum level within 1 hr. However, 20% of the substrate protein still remained unmodified after a 5-hr treatment.

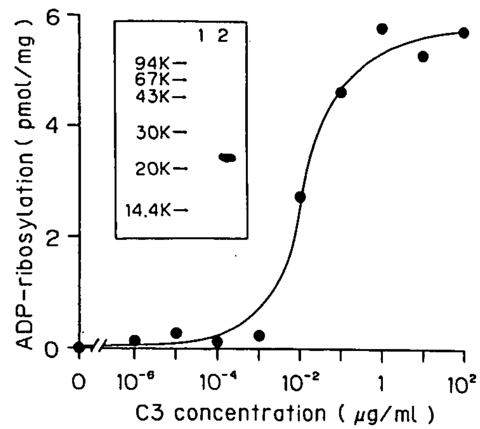

Fig. 8. ADP-ribosylation of proteins in GOTO cells by botulinum C3 exoenzyme. Dose-response curve for ADP-ribosylation. Inset: autoradiogram of proteins from GOTO cells that had been treated with C3 exoenzyme (lane 1, no enzyme; lane 2, C3 exoenzyme 50 ng/25 μg of cell lysate).

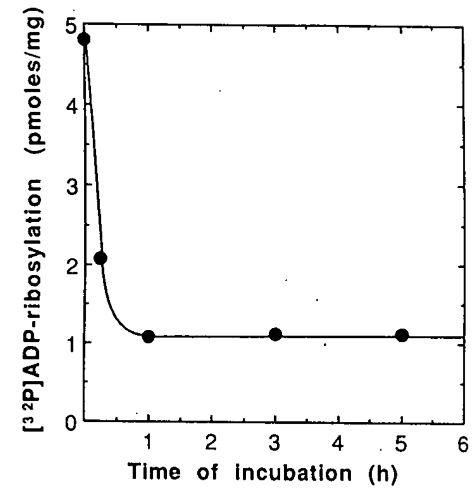

Fig. 9. Time course of ADP-ribosylation in GOTO cells by botulinum C3 exoenzyme. GOTO cells were treated with C3 exoenzyme at 3 μg/ml for the indicated times. Then the cells were lysed and an assay of ADP-ribosylation was carried out with C3 exoenzyme (50 ng/25 μg cell lysate) in the presence of [^{32}P]NAD. See "Materials and Methods" for details.

Discussion

Some of the effects of botulinum ADP-ribosyltransferase, C3 exoenzyme, on cultured cells were examined. Morphological effects of C3 exoenzyme have been reported for various lines of cells (5, 14, 17, 21). In this study, we determined the susceptibility of six lines of cultured cells to C3 exoenzyme under identical experimental conditions. C3 exoenzyme changed the morphology of cells in all six cell lines, which showed differences in susceptibility. Among the tested lines, neuroblastoma GOTO cells were the most susceptible to C3 exoenzyme. Condensation of the cytosol and the appearance of dendrites were marked. The dendrites resembled neurite nets. Formation of short neurites was report-

ed in PC-12 cells that possess neuron-like characteristics (14). To confirm its neurotropic activity, C3 exoenzyme was tested the neurite-outgrowth assay with chick ganglia, which has been the principal method for determining the activity of nerve growth factor (7). C3 exoenzyme evoked the outgrowth of neurites from the chick ganglion, as did nerve growth factor. In the case of PC-12 cells, C3 exoenzyme induced acetylcholine esterase activity (14). It could be, therefore, said that C3 exoenzyme is a neurotropic agent. However, a critical question remains to be answered before we can validly consider C3 exoenzyme to be a neurotropic factor: why is such a high dose necessary for induction of the outgrowth of neurites. As reported elsewhere, nerve tissue contains large amounts of substrate proteins (products of *rho* gene) for C3 ADP-ribosyltransferase (11). Therefore, we investigated the incorporation of C3 exoenzyme into GOTO cells and the correlation between the incorporation of C3 exoenzyme and its ability to alter the morphology of GOTO cells via ADP-ribosylation of the substrate proteins.

C3 exoenzyme was easily radioiodinated by chloramine T method without any loss of activity. ^{125}I-labeled C3 exoenzyme had the same neurite-inducing activity as the original unlabeled C3 exoenzyme. The amount of ^{125}I-labeled C3 exoenzyme taken up by GOTO cells was measured by an immunoprecipitation technique after treatment of cells with trypsin to liberate C3 exoenzyme associated with the cell membranes. ^{125}I-labeled C3 exoenzyme was quantitatively recovered from GOTO cells, with the amount depending on the duration of incubation. As shown in Fig. 4, GOTO cells started to change shape within 1 hr in the presence of C3 exoenzyme at 3 μg/ml. After a 1-hr incubation and washing, only 40 to 50% of GOTO cells changed shape within the next 24 hr. When GOTO cells were pulse-treated for 5 hr with C3 exoenzyme, all cells changed shape. In our experiments, approximately 8×10^8 molecules of C3 exoenzyme were present per GOTO cell but only 15–16 molecules of C3 exoenzyme were entrapped by each GOTO cell. Because of the low frequency of entry of the exoenzyme molecules into cells, a high dose of C3 exoenzyme is necessary to induce the morphological changes in GOTO cells. When GOTO cells were treated with C3 exoenzyme at 4C, the extent of morphological change and of the incorporation of C3 exoenzyme was depressed. These results strongly suggest that incorporation of C3 exoenzyme occurs by temperature-depending nonspecific endocytosis, for example, by simple diffusion, and not by specific receptor-mediated endocytosis.

The incorporated C3 exoenzyme seemed to be stable since SDS-PAGE and autoradiography showed that it retained its authentic size in the cytosol fraction of GOTO cells. Once C3 exoenzyme had been incorporated into GOTO cells, ADP-ribosylation of the substrate proteins was rapidly initiated and probably resulted in change in cell shape. ADP-ribosylation appeared to be necessary at a high level for at least 5 hr to induce the formation of neurites in GOTO cells. Previous observations indicate that the Rho proteins are involved in signal transduction and various cellular functions (4). ADP-ribosylation in GOTO cells is associated with the outgrowth of neurites, as described herein. However, as shown in Fig. 9, some unmodified substrate proteins were retained after induction of the change in cell shape. Some other event(s) may be required for completion of the process of neurite outgrowth in GOTO cells. C3 exoenzyme induces dysfunctional changes in the cytoskeleton of cultured cells (5, 7, 21). We are now performing experiments to monitor the cytoskeleton in GOTO cells that had been treated with C3 exoenzyme to determine how cytoskeletal proteins are related to the induction of the outgrowth of neurites. Functional alterations, such as changes in enzymatic activities related to properties of neuronal cells, will also be analyzed in GOTO cells.

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